

Review

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Tumor antigens for cancer immunotherapy: therapeutic potential of xenogeneic DNA vaccines

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Abstract

Preclinical animal studies have convincingly demonstrated that tumor immunity to self antigens can be actively induced and can translate into an effective anti-tumor response. Several of these observations are being tested in clinical trials. Immunization with xenogeneic DNA is an attractive approach to treat cancer since it generates T cell and antibody responses. When working in concert, these mechanisms may improve the efficacy of vaccines. The use of xenogeneic DNA in overcoming immune tolerance has been promising not only in inbred mice with transplanted tumors but also in outbred canines, which present with spontaneous tumors, as in the case of human. Use of this strategy also overcomes limitations seen in other types of cancer vaccines. Immunization against defined tumor antigens using a xenogeneic DNA vaccine is currently being tested in early phase clinical trials for the treatment of melanoma and prostate cancers, with proposed trials for breast cancer and Non-Hodgkin's Lymphoma.

Introduction

Since the 1990s, tumor immunology has developed into a distinct discipline with a metamorphosis from clinical observations in oncology to understanding its scientific underpinnings. This has been particularly relevant to the development of active immunotherapies (vaccines) for cancer. Traditionally, vaccines have been effective in the induction of protective immunity to bacteria and viruses based on recognition of foreign, or non-self, antigens on these pathogens. However, cancer cells arise from one's own tissue (self) and this poses a challenge in the development of effective active immunotherapies for cancer. It also presents a conundrum: can the immune system mount an effective response to reject tumors?

Perhaps the answer to the above question lies in the paradigm that the immune system can distinguish self from

'altered self' rather than the traditional non-self [1]. While some mutated gene products (altered self) have been identified, surprisingly, the vast majority of antigens on cancers characterized to date are unaltered self antigens. These are antigens encoded by genes expressed by both tumor cells as well as their normal cell counterparts. That cancer immunity exists, is observed clinically in the form of spontaneous regressions in melanoma, GI tumors, lung and breast cancers [2]. In addition, histopathology of tumor sections has revealed infiltrating lymphocytes around the tumor bed and recent studies indicate that ovarian cancer patients with such infiltrates around tumors have an improved prognosis, compared with similarly staged patients without lymphocytic infiltrates [3]. The immune repertoire therefore contains auto-reactive immune cells that may reject tumors, when activated appropriately. These auto-reactive cells, upon recognizing

target molecules on normal cells have the potential to induce tissue destruction leading to toxic autoimmunity.

In this article, we will outline the different types of antigens and their potential use in cancer immunotherapy. Secondly, we will summarize the data on immune responses generated to various proteins found on melanoma cells with an example of how differentiation antigens can be used as immunologic targets. In this section, we will describe the development of xenogeneic DNA vaccines from an initial laboratory concept into products used in human clinical trials. In the process, we will also highlight the results from an early clinical trial done in a new type of model with spontaneous cancer in outbred companion animals. This development of more predictive pre-clinical models may help to narrow the gap between very promising results seen in inbred animals with transplanted tumors and the relatively disappointing results obtained to date using cancer vaccines in humans.

Tumor antigens

The molecular characterization of several tumor antigens identified by both by T cells [4] and serology [5], has provided several candidates for the development of immunotherapy of various malignancies. Tumor antigens can be broadly categorized into two types – those that are undefined and others that are well defined.

Undefined antigens

Undefined and unidentified antigens are found in both allogeneic and autologous vaccine settings described below. Prominent examples of this type of vaccine based on undefined antigen are intact cells, cell lysate, total (amplified) RNA vaccines and heat-shock proteins. The underlying principle is that relevant tumor rejection antigens would be present among the thousands of other molecules that would be injected at the same time. The presence of unique as well as universal (or shared) tumor antigens in the mixture would prevent the expected emergence of antigen loss or escape variants. Tumors are known to commonly downregulate or lose key molecules to escape immune surveillance [6]. Therefore, use of vaccines with numerous targets that induce multiple components of the immune response is advantageous.

Some of the earliest attempts in inducing an anti-tumor response were seen in melanoma where intact, allogeneic cell lines were used as a vaccine. Allogeneic tumor vaccines may amplify the immune response as a result of non-specific stimulation. In addition, professional antigen presenting cells (APCs) such as dendritic cells (DCs) may phagocytose apoptotic tumor cells from the vaccine and effectively cross prime T cells with a host of immunogenic epitopes [7]. Canvaxin™ (CancerVax Corporation, CA), a whole cell vaccine, is a mixture of 3 sublethally irra-

diated, allogeneic melanoma lines, with different HLA haplotypes expressing various known tumor antigens. Early phase clinical trial results have shown that this vaccine induces both strong cellular DTH and high anti-TA90 IgM and anti GD2, GD3, GM2 and GM3 ganglioside IgM titers in patients with resected melanoma that are associated with improved survival [8]. Serum complement dependent cytotoxicity for melanoma cell lines in vitro also increased over baseline levels when patients were administered this polyvalent vaccine [9]. Having demonstrated encouraging response rates and low toxicity in Phase I and II trials, Canvaxin™ is presently being tested as a postsurgical adjuvant therapy in Phase 3 trials for AJCC stage III and IV melanoma [10], [11]. The vaccine was also tested in a small group of colorectal carcinoma patients with stage IV disease [12]. The rationale behind this was that shared antigens between melanoma and colorectal carcinoma would induce an anti-tumor response against colon carcinoma. Indeed, DTH responses to Canvaxin™ increased significantly in 78% of these patients after treatment. In addition, both IgM and IgG responses to TA90 were also elevated post treatment, with a correlation of higher IgM titers and disease free survival.

Allogeneic cell lysate vaccines provide a similar concept except that protein and other cellular components from the lysate serve as the immunogens. Melacine® (Corixa Corporation, WA) a lysate from two allogeneic melanoma lines plus an immunological adjuvant DETOX® (Corixa Corporation, WA) is the second type of allogeneic whole cell vaccine. This is also used in an adjuvant setting in resected Stage II melanoma patients [13]. Melacine has demonstrated a modest anti-tumor activity in AJCC stage IV melanoma, leading to licensure in Canada for use in advanced disease [14]. Clinical activity in the adjuvant setting may be more pronounced in patients expressing HLA-A2 and/or HLA-C3, with these patients showing significant improvements in relapse-free survival [15]. Prospective randomized trials are needed to confirm the clinical benefit of this type of vaccine in these HLA subsets in the adjuvant setting.

A third source of undefined tumor antigens is in the form of total tumor RNA (sometimes amplified when tumor availability is low) loaded onto dendritic cells (DCs), thus allowing for endogenous expression of total protein by a professional APC. Proof of principle was established using defined antigens in mouse models [16]. Early clinical trials using defined RNA antigens such as CEA [17] and PSA [18] showed induction of specific cytolytic CD8⁺ T cell responses. Subsequently, universal antigens such as telomerase [19] and survivin were used to show specificity in different tumor systems [20]. This idea has since evolved into a potential therapeutic approach using total tumor RNA, representing the entire pool of antigens. Vaccination

Table 1: Advantages and disadvantages of vaccines with defined and undefined antigens

	Advantages	Disadvantages
Undefined Antigens	<ol style="list-style-type: none"> 1. Availability of several potential tumor rejection antigens. 2. Unrestricted HLA patient population. 	<ol style="list-style-type: none"> 1. Difficulty in correlating clinical response and overall immune response based on select known antigens. 2. Largely dependent on clinical endpoint.
Defined Antigens	<ol style="list-style-type: none"> 1. Temporal monitoring of specific immune response. 2. Possibility of correlation of immune response with antigen expression on tumors. 	<ol style="list-style-type: none"> 1. Limited number of known tumor antigens for use (single or cocktail). 2. Relatively limited targeting of patient population due to HLA restriction.

Table 2: Classification of defined immunogens in cancer

Antigens	Examples	References
Unique antigens (usually caused by mutations)	p53, ras, β -catenin, CDK4, CDC27, α actinin-4	32, 34, 36, 37, 38, 39
Differentiation antigens	Tyrosinase, TRP1/gp75, TRP2, gp100, Melan-A/MART1, gangliosides, PSMA	42, 43, 44, 48, 50, 51
Overexpressed antigens	HER2, WT1, EphA3, EGFR, CD20	55, 59, 60, 61, 62
Cancer-testis antigens	MAGE, BAGE, GAGE, NY-ESO-1	63, 64
Universal antigens	Telomerase, Survivin	19, 20

with this type of product elicited T cell responses in leukemia and glioma [21], [22]. CD8⁺ specific reactivities against a broad set of tumor-associated antigens, including telomerase reverse transcriptase (TERT) were also seen in a clinical trial performed in patients with renal cell carcinoma [23], [24].

Heat-shock proteins (HSPs) have recently been demonstrated to be a means to generate an anti-tumor response by presenting the entire antigenic blueprint to the immune system. HSPs are expressed by cells in response to physical, chemical and environmental stress and are highly conserved in evolution. Immunologically, they function as intracellular peptide carriers and the complexes are taken up by DCs and macrophages to activate CD8⁺ cells [25], [26], [27]. In many instances, the HSP-peptide complexes are purified from an individual patient's tumor, whereas an alternative approach utilizes recombinant HSP linked to synthetic peptides [28]. Studies using HSP-peptide complexes in various early clinical trials indicate safety but with immunologic specificity and correlated clinical activity limited to a few patients with melanoma and colorectal carcinoma [29], [30], [31].

A vaccine source that provides the entire antigenic repertoire to the immune system has several advantages, perhaps the most important being a lack of HLA restriction among candidate patients. The drawback, however, is that there are only a few known tumor antigens that may be used as targets to monitor specific immune responses dur-

ing a trial, with most of them being weak antigens. As a result, it would be difficult to draw a correlation between an overall immunologic response to multiple antigens and a clinical outcome (Table 1). The search for new markers and optimum methods to monitor immune responses could perhaps bridge the two and help to improve vaccine design and efficacy for the next generation of this type of vaccine.

Defined antigens

An advantage of using defined antigens for immunotherapy is the ability to correlate specific immune responses with the antigen used, thus providing a means to study and improve immunogenicity of the vaccine, though the vaccine will have to be targeted to patients of selected HLA types. The approach of using defined antigens has been most widely explored in trials of individual antigens though combinations have also been tested. Using a 'cocktail' of defined antigens addresses some of the concerns about the emergence of antigen escape variants. These antigens can be grouped into various categories as indicated below (Table 2).

Unique antigens

Mutations in genes may create new gene products with or without altered gene function. These can give rise to new antigenic epitopes that may be immunogenic. Typical examples include proto-oncogenes that are involved in normal cell division and differentiation. A single point mutation may activate an oncogene (e.g. *ras*, *b-raf*) or

inactivate tumor suppressor genes (CDK4, p53) causing increased signal transduction and uncontrolled cell division. These mutations result in proteins that are involved in the induction of malignancy and may be important in maintaining the malignant phenotype, making them compelling targets for immunotherapy. Point mutations in tumor suppressor genes such in p53 are seen in about 50% of human malignancies. Antibodies to p53 mutations correlate with poor prognosis and can be associated with either undetected malignancy or a pre-malignant state [32]. Mutations in the *ras* gene occur in approximately 15% of cancers and the gene product has been shown to induce both CD4⁺ and CD8⁺ T cell responses in colorectal and pancreatic cancers [33], [34], [35]. In melanoma patients, mutations in β -catenin, a protein involved in cell adhesion and signaling regulation, result in epitopes that induce a HLA-A*24 restricted CD8⁺ T cell response [36]. Similarly, an amino acid substitution in CDK4, a cell cycle regulator, is recognized by CD8⁺ T cells [37]. A mutation in CDC27 causing altered protein trafficking into the endosomal compartment was found in a melanoma. This allows for the presentation of an MHC class II epitope and recognition by CD4⁺ cells [38]. More recently, CD8⁺ T cells recognizing a mutated decapeptide of α -actinin 4 were isolated from a human lung cancer [39]. Accumulation of α -actinin 4 in the cytoplasm causes actin bundling which increases cellular motility and may contribute to metastasis [40].

Differentiation antigens

Tissue specific differentiation antigens are molecules present on tumor cells and their normal cell counterparts. Melanoma has been an excellent model to study differentiation antigens as tumor targets for immunotherapy, given the relative restricted expression of the proteins involved in melanin biosynthesis. The prototype of this family of differentiation antigens is tyrosinase, the rate-limiting enzyme in melanin synthesis. Tyrosinase Related Protein-1 (TRP)-1/hgp75, which may have DHICA oxidase activity, stabilizes tyrosinase. Serum IgG antibodies that immunoprecipitate TRP-1 have been identified in melanoma patients [41], suggesting that other members in the melanin synthesis pathway may also be recognized by the immune system. A variety of Class I and II binding epitopes have been identified in the sequences of tyrosinase, TRP-1/gp75, TRP-2 and gp100/pmel17 [42], [43], [44]. Immune responses to these antigens are mediated by various effector mechanisms. Tumor protection in mice in response to TRP-1/gp75 vaccination is antibody dependent, whether by passive [45] or active immunization [46], [47]. Melan-A (MART-1), whose function is not clear, is another melanosomal protein for which CD8⁺ T cells have been identified [48]. The other group of differentiation antigens that is overexpressed is comprised of gangliosides (GM3, GM2, GD2, GD3). GD3, a representative of

this family, is a glycolipid with an extracellular carbohydrate moiety consisting of negatively charged sialic acid residues, which are immunogenic. The entire molecule is anchored into the lipid cell membrane by a hydrophobic ceramide backbone [49]. GM2 is the most immunogenic ganglioside with higher antibody titres correlating with better prognosis [50].

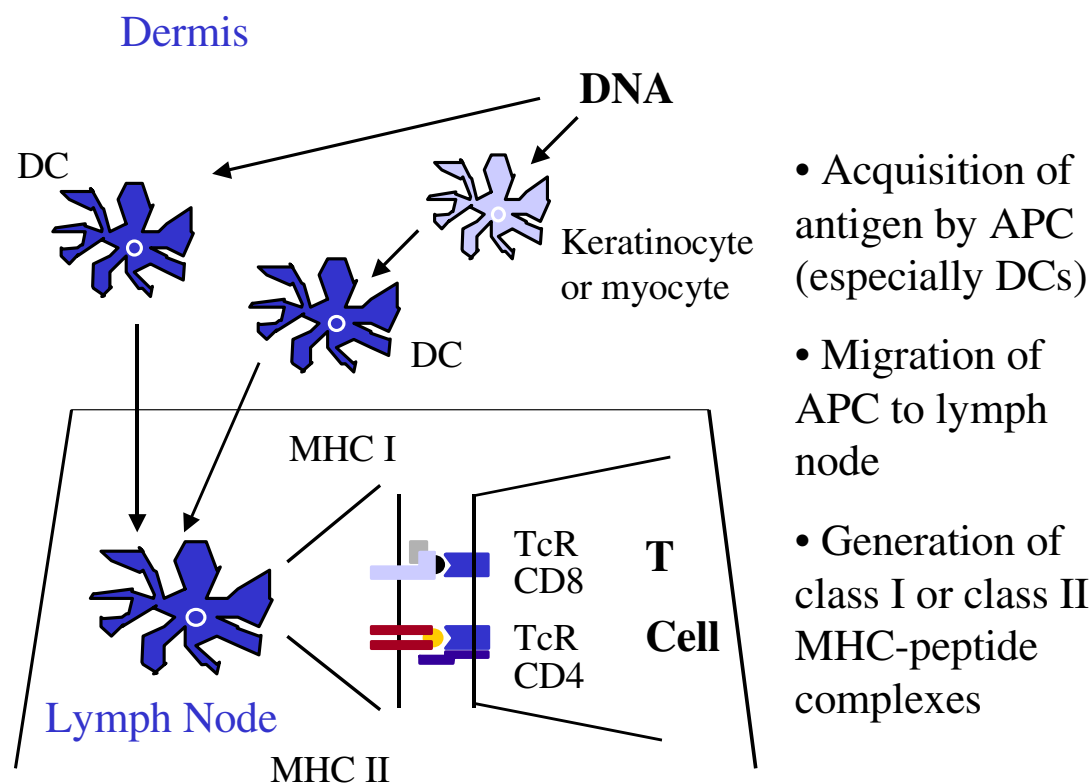
Expression of antigen targets for active immunotherapy is certainly not limited to melanoma cells. Other solid tumors, such as prostate cancer have proteins that would make reasonable vaccine targets, such as prostate specific membrane antigen (PSMA) [51]. Prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) also form good targets for the treatment of prostate cancer, though expression is not restricted to this cancer [52].

Overexpressed antigens

Overexpressed antigens on cancers form attractive targets as an immune response to tumors can be elicited while reducing potential autoimmune attack on cells bearing the normal copy number of genes. Her2/*neu* (HER2) is a proto-oncogene which shares homology with other members of the HER family of tyrosine kinase receptors and the epidermal growth factor receptor [53]. The cell surface glycoprotein is overexpressed in about 30% of breast cancers associated with disease aggression and poor prognosis [54]. HER2 expression is also seen in ovary, lung, pancreas, prostate and colon cancers. Anti-tumor effects of Trastuzumab (Herceptin® – a humanized monoclonal antibody) in breast cancer are mediated by either inducing apoptosis [55] or Fc receptor mediated cellular antibody-dependent cytotoxicity [56]. However, response to this treatment, though encouraging, is limited to a small percentage of advanced stage patients. In an attempt to induce both T cell and antibody responses Foy et al [57] showed the effectiveness of developing a deleted variant of HER2 (dHER2). This protein lacks the transmembrane and the kinase region of the intracellular domain (ICD), retaining just the extracellular domain (ECD) and the carboxyl terminal of the autophosphorylation domain of the ICD and is effective in generating an anti tumor response in mice [58]. Wilm's Tumor 1 (WT1) and Ephrin receptor (Eph3) proteins are also overexpressed proteins and are examples of targets for active immunotherapy [59], [60], while epidermal growth factor receptor (EGFR) and CD20 are overexpressed on colorectal cancer and lymphomas respectively and are examples of targets for passive antibody immunotherapy [61], [62].

Cancer testis antigens

This class of antigens is found on male germ cells and is silent on healthy somatic cells, but expressed on a variety of tumors. After the identification of MAGE-1 [63], which was shown to induce a CD8⁺ response, several antigens

**Figure 1**

Methods of antigen presentation that could generate an immune response after DNA immunization. DNA can directly transfect dendritic cells (DCs) which can migrate to the draining lymph node to activate naïve T cells. Alternately, they can be cross primed when they uptake antigen from dying keratinocytes or myocytes. They can activate both CD8⁺ and CD4⁺ cells in the lymph node via Class I or Class II peptide-MHC complexes. Abbreviations – DCs dendritic cells, APC Antigen presenting cells, MHC Major Histocompatibility Complex, TcR T Cell Receptor Complex

from related families have been identified (MAGE, BAGE, GAGE). NY-ESO-1, unrelated to the other members mentioned, is also a germ cell antigen, with both Class I and II restricted epitopes [64], [65]. With restricted expression on normal tissues, this group of antigens would potentially lead to fewer and probably less severe autoimmune reactions.

DNA vaccines

The immunogenicity of antigens delivered via plasmid DNA was first seen in viral studies, where cDNA encoding an influenza viral protein generated specific cytotoxic T cells that could protect against a live influenza viral challenge [66]. In a plasmid DNA vaccine, the gene of interest is cloned into a bacterial expression vector having a con-

stitutively active promoter for expression of the gene product. The plasmid can be introduced into the dermis or muscle where it is taken up by professional antigen presenting cells (APCs) such as dendritic cells (DCs) as well as by neighboring non-APCs and can be expressed for up to two months [67]. One of two methods of uptake is possible (Fig. 1). The first possibility is the direct transfection of APCs by plasmid DNA [68]. Even though a relatively small number of cells present at the vaccination site are DCs, their enhanced potential to present and prime T cells can make this feasible. The second mechanism underlying the efficacy of DNA immunization is cross priming [69], [70], [71]. The DNA transfects neighboring keratinocytes or myocytes that transcribe and translate the antigen. Mature antigen is made available to DCs as secreted pro-

tein or through apoptotic transfected cells. The antigen is then processed and presented to naïve T cells in draining lymph nodes.

DNA vaccines have some properties that help to overcome obstacles encountered with the use of other types of cancer vaccines. Dendritic cells as APCs for peptides, proteins or RNA are known to be effective in generating antigen specific responses [72]. However, in a clinical setting, autologous cellular vaccines must be custom manufactured for each patient, making them cost prohibitive and labor intensive in a large vaccine trial. Peptide vaccines, while being simpler to manufacture, can be effective only in association with certain HLA molecules. Consequently, only a limited pool of patients bearing the appropriate HLA type is eligible to receive the vaccine. Though immune monitoring to these vaccines is more straightforward, the potential for antigen escape variants is greater, as tumors theoretically only need to alter a single amino acid to abolish presentation of a given epitope. Protein vaccines, on the other hand, are not HLA restricted and can present a variety of epitopes to activate both cell mediated and humoral arms of the immune system. However, large scale manufacturing, which includes purification, can be a challenge.

DNA vaccines encoding full length protein can circumvent some of these problems while having the advantages of purified recombinant protein. First, full length cDNA of the gene of interest provides several potential epitopes to stimulate both cytolytic T cells as well as an antibody response, the latter indicating the presence of strong helper epitopes in the gene sequence. Second, insertion of the antigen coding sequence in a bacterial expression vector provides the vaccine with a 'built-in adjuvant' offered by unmethylated CpG motifs [73], [74]. Third, transcribing and translating the full length protein also eliminates the need to limit patients of a defined HLA type to be eligible to receive the vaccine. The simplicity and relative economy of producing large quantities of DNA (versus purified recombinant protein) also makes this approach attractive. More importantly, DNA vaccines in human trials for malaria and HIV treatment have shown that they are well tolerated and safe [75], [76], [77], [78], [79]. An added benefit is the relative ease to design and produce altered forms of the wild type antigen with higher biological potency.

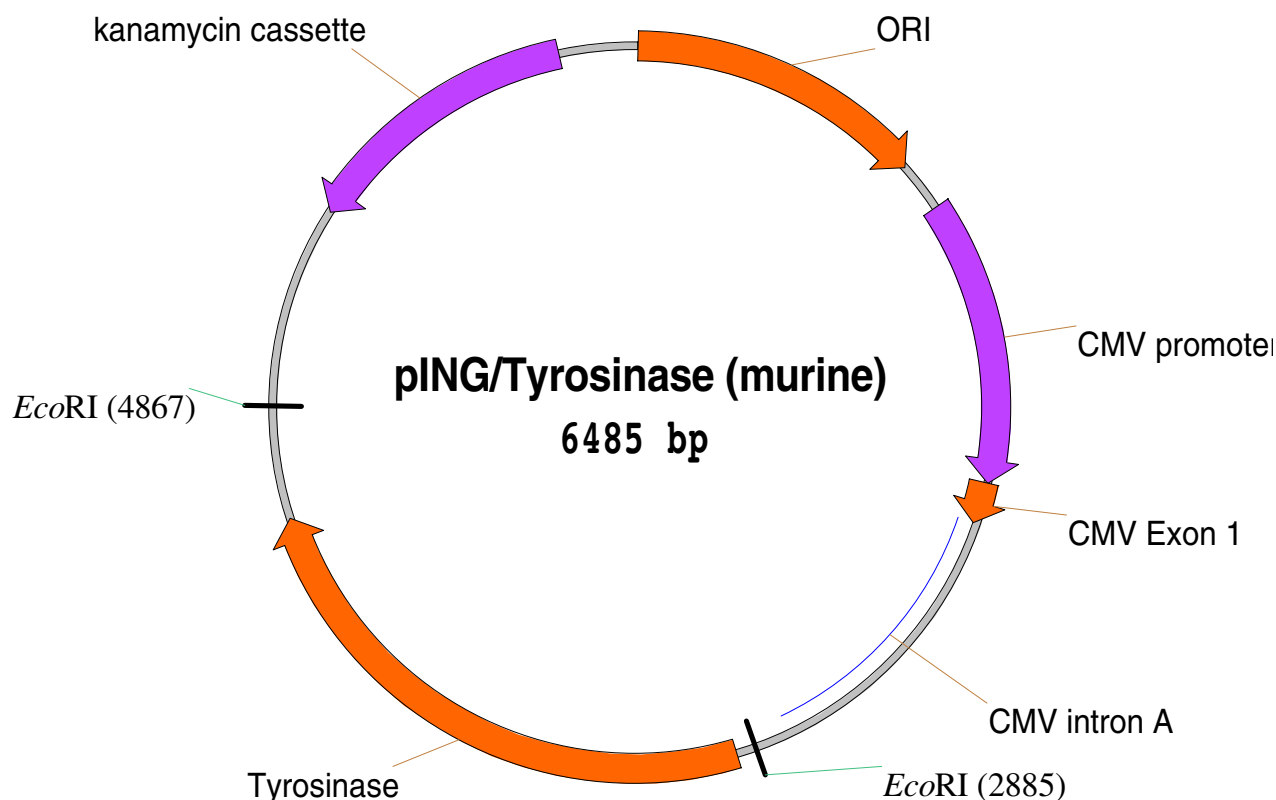
Murine studies to support the use of xenogeneic immunization

The importance of using an 'altered self' form of antigen to induce tumor protection came from studies using lysates of SK-MEL19, a gp75⁺ human melanoma cell line [46]. When mice were immunized with human melanoma lysate, autoantibodies that recognized mouse

gp75 were produced. Immunization with murine B16 melanoma produced no antibody response, even when potent adjuvants were included. These studies support the idea that ignorance or tolerance to a self protein can be overcome by presenting sources of altered antigen (e.g., homologous xenogeneic protein). A similar study in a rat Her2/neu model, showed that immunization with human intracellular domain segment of the protein generated T cell and antibody responses specific for both rat and human Her2/neu [80]. This indicates that despite extensive homology between the mouse and human protein, small differences in epitopes between the two are sufficient to overcome immune ignorance or tolerance.

This idea was further tested with a variety of melanosomal differentiation antigens, starting with human TRP1/gp75 [47]. Human gp75 cDNA, expressed in a plasmid expression vector and introduced into the epidermis via gene gun, protected mice from a syngeneic B16 tumor challenge primary through autoantibodies, while syngeneic (murine) gp75 induced no tumor immunity. Tumor protection required Fcγ receptors (FcγR), CD4⁺ cells and NK1.1⁺ cells, but interestingly was independent of CD8⁺ T cells [47]. In addition to protection from tumor challenge, many of the mice immunized with human gp75 DNA also developed hypopigmentation of coat, presumably through cross-recognition of endogenous gp75 on melanocytes in the mouse hair follicle. An example of the expression plasmid containing murine tyrosinase DNA vaccine is shown in Fig 2. This vaccine is currently being used in a clinical trial at Memorial Sloan-Kettering Cancer Center, New York.

In tumor protection studies using the other melanosomal antigens, a similar requirement for the xenogeneic antigen was noted; however, there were significant differences in the immunologic mechanisms underlying the tumor immunity. TRP2, another protein in the melanin synthesis pathway led to a potent induction of CD8⁺ T cells and required both CD4⁺ and CD8⁺ effectors for tumor protection [81]. There was no dependence on antibodies or NK1.1⁺ cells in this case. Gp100, another melanosomal protein, conferred tumor protection through CD8⁺ T cells, though without a strict requirement for CD4⁺ help [82]. While these immunogens were effective in a prophylactic setting, a 'treatment' model to mimic the clinical scenario was also tested. Using TRP2 as the antigen, two models were tested. In the first case, immunization was started 10 days after injecting live tumor intravenously [81]. In the second case, B16 melanoma was given orthotopically in the foot pad and then surgically excised. Immunization with huTRP2 was then carried out in a 'minimal residual disease' setting that is comparable to adjuvant therapy for micrometastatic cancer. A significant decrease in the

**Figure 2**

Plasmid DNA expressing mouse tyrosinase used in clinical trials at MSKCC. The full length murine tyrosinase cDNA was cloned into a bacterial expression vector having a kanamycin resistance cassette and operating under the host's constitutive CMV promoter for expression

development of lung metastases was noted after immunization with human TRP2 [83].

Epitope spreading was an interesting phenomenon observed among mice immunized with huTRP2 DNA. In some of the mice, anti-TRP2 antibodies were also specific to gp75, a related protein [84]. Determinant spreading is a normal feature of protective immune responses to infectious agents, allowing recognition of multiple antigenic targets [85]. While the immune system depends on diversification to adequately protect against non-self, it is possible that it may also play a role in protection against aberrant processes that are dangerous to 'self', such as cancer. Epitope spreading (both intermolecular and intramo-

lecular) was noted in few cases of clinical responders to peptide vaccines in trials involving patients with melanoma. When immunized with MART-127-35 loaded DCs, one patient developed HLA-A*0201-restricted responses to two additional melanoma antigens (gp100 and tyrosinase) as well as a HLA-DR4-restricted MART-1 epitope [86]. The patient's tumor was positive for MART-1, gp100 and tyrosinase. In another instance of inter- and intramolecular spreading, the patient (who was a responder), was immunized with DCs loaded with HLA-A*0201 melanoma-derived epitopes MART1₂₇₋₃₅, gp100₂₈₀₋₂₈₈, and tyrosinase_{368-376D}. The patient's T cells showed reactivity to two other HLA-A*0201-binding epitopes (gp100₂₀₉₋₂₁₇ and tyrosinase₁₋₉) and four HLA-

DR4 class II epitopes (MART1₅₁₋₇₃, gp100₄₄₋₅₉, gp100₆₁₅₋₆₃₃, and tyrosinase₅₆₋₇₀) [87]. Temporary regression of a melanoma metastasis was also associated with immune reactivity toward a cryptic epitope from the MAGE-12 gene (MAGE-12₁₇₀₋₁₇₈), after being immunized with gp100_{209-2M} [88]. Spreading of immune reactivity to other melanoma antigens in subjects with clinical response, suggests that determinant spreading may be associated with clinical response to immunotherapy.

In early human clinical trials for infectious diseases, DNA vaccines have not been as potent as might have been expected given pre-clinical mouse studies [76], [78]. Several studies have shown the benefit of adding cytokines such as GM-CSF (both DNA and soluble protein) to enhance the antigen specific response, perhaps by mobilizing DCs as well as enhancing expression of co-stimulatory molecules [89], [90], [91], [92]. DNA encoding GM-CSF was shown to improve recruitment of DCs to the local site of injection [89]) as well as to induce infiltration of inflammatory and Th1 precursors cytokines [90]). Co-immunization of full length rat *neu* cDNA with plasmid DNA coding for co-stimulatory molecules such as CD80, CD86, and CD137 in a rat transgenic mouse model induced both antigen specific T cells and antibodies resulting in an anti-tumor effect [92]. Local use of GM-CSF DNA can abrogate the inconvenience of multiple injections of soluble GM-CSF protein, while potentially offering the same benefits.

Development of xenogeneic DNA Vaccines for use in canines with spontaneous cancer

As mentioned above, immunotherapies that appear promising in pre-clinical mouse models have often led to clinical trials with disappointing clinical and immunological results. A study was conducted in collaboration with the Animal Medical Center of NY, a tertiary care hospital for pets that has an oncology clinic that sees up to 5000 visits per year. The use of outbred animals with spontaneously arising malignancies may overcome some of the limitations of transplantable tumor systems in syngeneic mice and serve as a translational bridge between standard inbred animal models and human clinical trials.

In dogs, malignant melanoma of the oral mucosa displays a similar natural history to human cutaneous melanoma. This includes early invasion, a predisposition to distant metastasis and relative resistance to standard cytotoxic therapies. Radical surgery followed by radiation is optimal therapy; however, local and distant recurrence is common and difficult to treat. An initial clinical trial using human tyrosinase DNA in 9 dogs with metastatic melanoma was recently completed [93]. The vaccine was given by the same route and at the same doses that are to be used in the human clinical trial. There has been no tox-

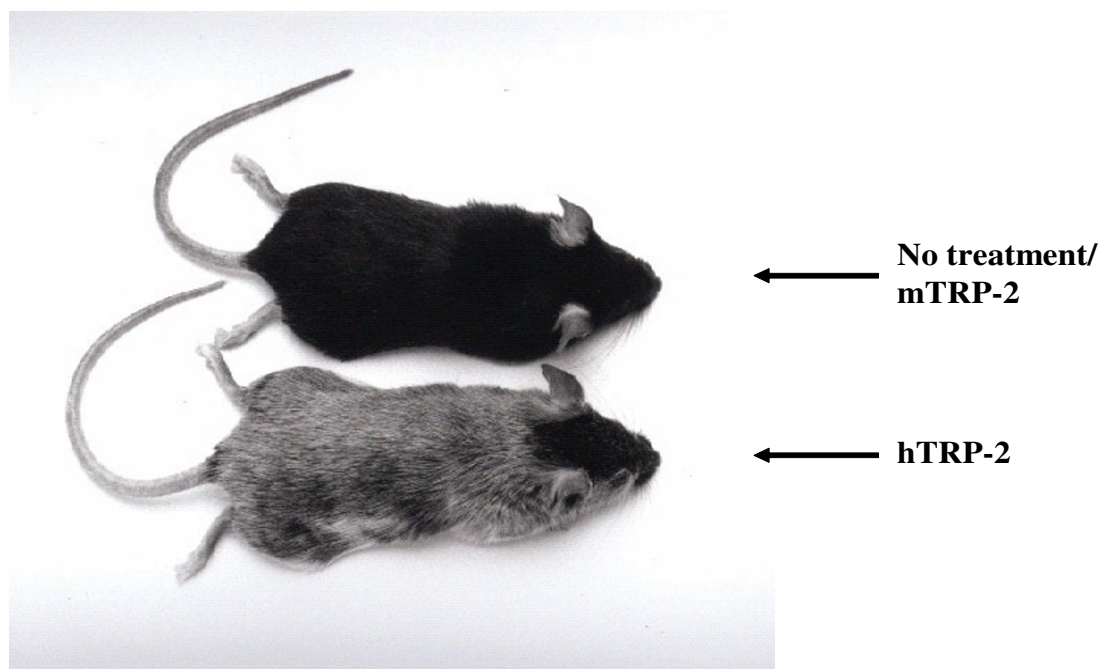
icity associated with the vaccination. In addition, one dog with numerous lung metastases has had a complete clinical response with disappearance of all detectable disease, lasting over one year. The median actuarial survival for dogs on this trial predicted by Kaplan-Meier analysis is greater than 389 days. Although this is a small single-arm study, this data is encouraging when considered in the context that stage-matched historical controls had a survival of less than 90 days. Similar trials have also been completed using murine tyrosinase and murine gp75 DNA in dogs with melanoma. Follow-up is too short at this time to reach any clinical conclusions for these trials. A trial of GM-CSF DNA alone or in combination with murine tyrosinase DNA is currently underway.

Clinical trials in human using syngeneic cDNA in cancer therapy

In a phase 1 safety study using syngeneic cDNA to CEA, low grade transient toxicity was observed [94]. While CEA-specific antibodies were not observed, 4 of 17 patients showed lymphoproliferative responses to CEA after vaccination. There was no association with objective tumor regression and sustained declines in circulating CEA, nor a correlation between lymphoproliferative response with stable disease. In another recent clinical trial, syngeneic cDNA encoding gp100 was used as the vaccine. The results did not demonstrate clinical or immunologic responses to the vaccine [95]. Several studies have indicated that syngeneic cDNA is immunogenic when used either in prime boost regimens with recombinant viral vectors or with the use of augmentation strategies such as cytokines or costimulatory molecules. The presence of slight differences in epitopes between host 'self' protein and that encoded by xenogeneic DNA plasmid vaccine, along with inherent bacterial unmethylated CpG motifs may be sufficient to boost the immune response to break tolerance to tumors.

Autoimmunity

Attempting to generate immune responses to self proteins raises reciprocal problems of immunological tolerance and potential autoimmune sequelae. In murine studies using melanosomal differentiation antigens, autoimmune depigmentation was commonly seen [45], [46], [81] (Fig 3). However, in trials of several immunologic therapies for cancer, autoimmune manifestations remain rare, despite induction of immune responses. A clinical study in melanoma using adoptive transfer of selected tumor reactive T cells showed that regression of metastatic melanoma was accompanied by autoimmune depigmentation [96]. However, effector mechanisms for both tumor immunity and autoimmunity could be different. Murine studies have shown that active immunization with human gp75 induces an antibody response that depends on activating FcγR I and/or III to reject tumor,

**Figure 3**

Autoimmune depigmentation as a result of immunization with human TRP2/DCT. Abbreviations – hTRP2 *human tyrosinase related protein-2*; mTRP2 *mouse tyrosinase related protein-2*

while depigmentation continued in the absence of this receptor [47]. Likewise, studies using knockout mice indicated that when TRP2 was used as the immunogen, autoimmunity was dependent on perforin, whereas tumor immunity proceeded in the absence of perforin [81].

Conclusion

It is now accepted that tolerance to self antigens on cancer cells can be overcome using active immunization strategies, such as with xenogeneic DNA vaccines. The hallmarks of a successful vaccine are judged by multiple endpoints, with the most important one being control of dissemination of tumor. There are several steps involved in the generation of anti-tumor immune responses. First, there must be efficient uptake of antigen by professional APCs, such as Langerhans cells and DCs, followed by anti-

gen processing and migration to draining lymph nodes. Precise antigen presentation, leading to induction and expansion of appropriate helper and cytotoxic cells bearing the cognate receptor is necessary. These effector cells must then traffic to distant tumor sites, recognize and lyse tumor. There should be a persistent memory pool of effectors to challenge tumors bearing the same antigen that might grow out over time. Ultimately, an adaptive response should be generated to control antigen escape variants. The potency of the response, once induced, must be increased to the magnitude of that as found in infectious disease settings. A break anywhere in this sequence can give rise to disease progression. Unfortunately, this is a frustration that is frequently encountered. Specific immune responses to tumor antigens in vitro can be detected in patients undergoing various immunotherapies that do not translate to a desired clinical response. A

major step forward in understanding and improving vaccine efficacy in cancer immunotherapy is the concordance of clinical outcomes with appropriate, well-timed and accurate immunologic monitoring.

The search for an active immunotherapy for cancer is clearly not easy. The xenogeneic DNA vaccine approach is only one among the several that has potential in treating cancer. Research in animal models (inbred mice and outbred companion animals) has shown great promise for this in the treatment of solid tumors. Based on these results, this immunization strategy is being tested in patients with melanoma and prostate cancers at MSKCC, NY, with further clinical trials proposed for breast cancer and Non-Hodgkin's Lymphoma.

List of abbreviations

Defined in the text

Competing interests

RS is a salaried employee of CancerVax Corporation, CA, with stock options.

The authors have neither financial nor non-financial competing interests.

Authors' contributions

RS conceived of the review and identified the sections within. JDW wrote the section on 'Development of xenogeneic DNA vaccines for use in canines with spontaneous cancer'. RS wrote the other sections of the manuscript. Both authors edited the draft, and read and approved the final manuscript.

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